Axon guidance: **A balance of signals sets axons on the right track**

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Axon guidance depends on the transduction of extracellular guidance cues into motile responses by the axonal growth cone. Recent studies *in vivo* have elucidated mechanisms required for this process that involve kinases and phosphatases, calcium dynamics and remodeling of the actin cytoskeleton.

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The development of the nervous system depends on the process of axonal navigation. Axons correctly interpret molecular guidance cues in their environment by transducing extracellular signals into characteristic patterns of axonal growth. The navigation of each axon is determined by the motile activities of its terminus, the growth cone. In turn, growth cone motility is dependent on the dynamic remodeling of the actin cytoskeleton. Therefore, guidance cues elicit appropriate motile responses from growth cones, at least in part, by regulating the dynamics and structure of the actin cytoskeleton. Recent investigations of axonal navigation *in vivo* have provided significant insights into the roles of various factors — receptor protein phosphatases, protein kinases, cytoplasmic calcium dynamics and intracellular regulators of the actin cytoskeleton.

Role of kinases and phosphatases

Many extracellular signals that control the direction of axonal outgrowth act through signal transduction pathways involving either receptor tyrosine kinases or cytoplasmic kinases that are activated downstream of ligand–receptor binding. *Drosophila* Abl is a cytoplasmic tyrosine kinase expressed in the developing axons of some neurons. In the first of their two recent reports published in *Neuron*, Wills *et al.* [1] investigated the role of Abl in axonal growth and found that Abl is required for the correct growth of a subset of *Drosophila* motor neuronal axons that arises from the intersegmental nerve (ISN), termed ISNb. Normally, ISNb axons defasciculate from the ISN branch point and innervate ventral muscles (Figure 1a). Using antibodies that specifically recognize motor neurons, Wills *et al.* [1] demonstrated that ISNb axons in Abl mutants exhibit a 'stalled' phenotype characterized by the premature arrest of growth cones at muscle cells past which these growth cones would normally grow (Figure 1a). Further, this ISNb phenotype of *Drosophila* Abl mutants was dependent on the kinase activity of Abl, because it could be rescued by increasing the expression of wild-type Abl but not a truncated form of Abl that lacked the kinase domain.

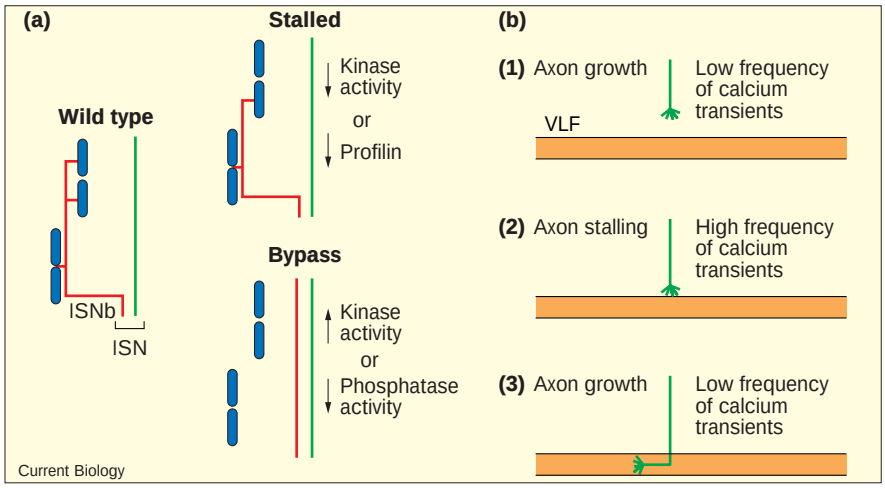
Dlar is a receptor tyrosine phosphatase expressed in some *Drosophila* developing neurons. In their second report, Wills *et al.* [2] generated *Drosophila* mutants lacking the expression of Dlar; in these mutants, ISNb axons failed to defasciculate and innervate their correct target muscles. Rather, these axons continued growing along the ISN — the so-called ISNb-bypass phenotype. The presence of a receptor phosphatase suggests that it acts to regulate the activity of one or more kinases. By biochemical analysis *in vitro*, Abl was found to physically interact with the cytoplasmic domain of Dlar and was dephosphorylated by Dlar [2]. In addition, antagonistic interactions between Dlar and Abl in regulating ISNb axonal growth were revealed by genetic studies [2]. Decreasing the expression levels of Abl largely decreased the severity of the phenotype resulting from the loss of Dlar, suggesting that lower levels of protein phosphorylation by Abl can compensate for the reduced protein dephosphorylation arising from the decreased Dlar activity. Conversely, increasing Abl levels in a wild-type genetic background reproduced the ISNb-bypass phenotype observed in Dlar-deficient mutants. Overexpression of a form of Abl that lacked the kinase domain, however, did not produce the ISNb-bypass phenotype, demonstrating the requirement for Abl kinase activity in achieving this effect. Finally, increasing the expression of both Dlar and Abl resulted in a normal ISNb phenotype.

Collectively, these data demonstrate that guidance of ISNb axons depends on the antagonistic effects of the activities of Dlar and Abl. Increases in Abl-mediated protein phosphorylation, resulting from either the loss of Dlar function or increased levels of Abl kinase, produce the ISNb-bypass phenotype. These results are very exciting in that they demonstrate for the first time that a balance of protein phosphorylation achieved by the combined activities of a kinase and a phosphatase determine specific aspects of axon guidance *in vivo*.

Reorganization of the actin cytoskeleton

Profilin is an actin-binding protein that has been implicated in regulating the dynamics and organization of the actin cytoskeleton. While studying the effects of removing Abl expression, Wills *et al.* [1] also examined the effects of the loss of profilin expression on axonal growth. They found that neurons of *Drosophila* embryos lacking profilin expression exhibited generalized defects in axonal extension without obvious alterations in growth cone morphology.

Figure 1



(a) A balance of phosphatase and kinase activity regulates the guidance of *Drosophila* ISNb axons. In wild-type embryos, the ISNb axons (red) defasciculate from the ISN and innervate ventral muscles (blue). In the stalled phenotype, the ISNb axons do not arrive at the distal-most muscle targets. In the ISNb-bypass phenotype, the ISNb axons do not defasciculate and continue growing along the ISN. (b) In *Xenopus* embryos, the frequency of intracellular calcium transients in spinal cord growth cones correlates with the behavior of the growth cones during a 90° turn to fasciculate with the axons of the ventral lateral funiculus (VLF; orange). (1) While axons grow towards the VLF, their growth cones exhibit a low frequency of calcium transients and a fast rate of growth. (2) Upon contact with the VLF, growth cones stall and exhibit a high frequency of calcium transients. (3) Following turning and fasciculation with the axons of the VLF, growth cones resume a fast rate of growth and exhibit a low frequency of calcium transients.

Experiments that examined axonal growth *in vitro*, by culturing explanted ventral nerve cords on a 'neutral' substratum, revealed that the axons from profilin-mutant flies extended only around two-thirds of the distance of wild-type axons. Profilin-deficient mutants displayed a striking similarity to the Abl-deficient mutants in that the ISNb axons exhibited a premature stalling phenotype. Further, Wills *et al.* [1] demonstrated that profilin-deficient flies that had only half of the normal levels of Abl expression exhibited even greater defects in axonal outgrowth than profilin mutants with normal levels of Abl. These data indicate that Abl and profilin are involved in the same signal transduction pathways during ISN axon growth *in vivo*.

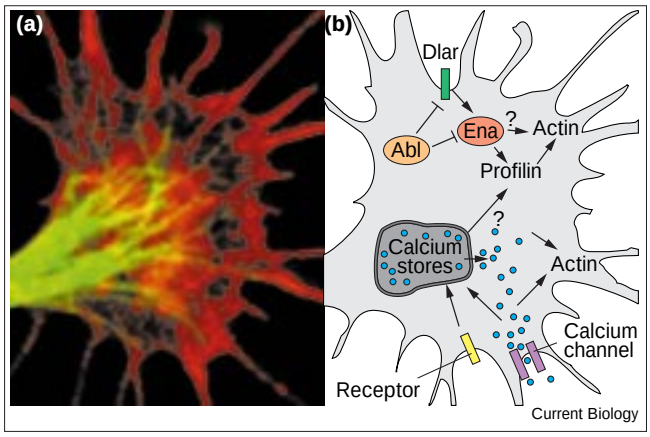
Drosophila Ena is a substrate of Abl that binds to profilin and has been implicated in the regulation of the actin cytoskeleton. In their second study, Wills *et al.* [2] also looked at the effects of the lack of Ena expression on axonal growth and established a link between Ena, Abl and Dlar. The authors found that the ISNb phenotype of Ena loss-of-function mutants resembled the ISNb-bypass phenotype of Abl gain-of-function mutants. Also, Ena associated directly with Dlar and was a substrate for this phosphatase. Multiple ISNb phenotypes were present in Ena-deficient mutants, however, suggesting that Ena may act as a convergence point for multiple signals during ISNb guidance. Dlar, Abl, Ena and profilin therefore cooperate in the growth and guidance of ISN axons, in particular ISNb axons, in *Drosophila* neurodevelopment.

The possibility that similar interactions operate in vertebrate axonal guidance was investigated by Lanier *et al.* [3] in mice with a targeted deletion of the mammalian ortholog of Ena, Mena. Mena-deficient mice exhibited deficits in formation of the corpus callosum, the major intercortical projection of the vertebrate nervous system that connects the two hemispheres of the telencephalon. In mice lacking Mena, axons projected towards the midline in a manner similar to wild type, but they failed to cross the midline and instead formed axonal tangles. The failure to form corpus callosum did not represent a generalized deficit in commissure formation, because other commissures — such as the anterior commissure — formed normally in the Mena-deficient mice. Importantly, Lanier *et al.* [3] demonstrated by *in vitro* analysis that Mena was localized to the tips of lamellipodia and filopodia of growth cones. Because filopodia and lamellipodia are necessary for growth cone guidance [4,5], this distribution of Mena is consistent with a role for Mena in regulating axonal guidance. Furthermore, interactions between Mena and profilin are also important during stages of neurogenesis prior to axonal guidance. Examination of Mena-deficient embryos that also expressed only half of the wild-type amount of profilin revealed lethal defects in neural tube closure [3]. In both *Drosophila* and mice, therefore, Ena and profilin interact in mediating important aspects of neurogenesis.

Involvement of calcium transients

On the basis of *in vitro* studies, it has been well established that intracellular calcium transients can regulate growth cone motility and axonal growth [6,7]. Further investigations into the role of growth-cone calcium transients in regulating the extension of multiple neuronal axon types in the developing spinal cord of *Xenopus* have been made by Gomez and Spitzer [8]. These authors reported that, while axons extended in the spinal cord, their growth cones

Figure 2



(a) Immunofluorescence image of a growth cone in which the actin filaments are stained red and microtubules green. (b) Diagram of the relationships between elements of the growth-cone guidance system. The transmembrane receptor phosphatase Dlar may alter profilin activity via Ena. The activity of profilin – and possibly Ena – in turn could regulate the dynamics and structure of the actin cytoskeleton. Abl may downregulate the activity of Dlar and Ena. Transmembrane receptors may regulate growth cone calcium transients by affecting the entry of calcium into the cytoplasm through either membrane calcium channels or by release from intracellular stores (such as the endoplasmic reticulum or mitochondria). Calcium entering the cytoplasm through membrane channels may also activate release of additional calcium from intracellular stores. Calcium could regulate the structure and dynamics of the actin cytoskeleton either directly or indirectly though a number of calcium-dependent actin-binding proteins.

exhibited calcium transients, and the occurrence of calcium transients correlated with periods of decreased axonal extension. Experimental inhibition of calcium transients, using membrane-permeable calcium chelators, resulted in faster rates of axonal extension. Conversely, experimental induction of calcium transients — by photolytically uncaging calcium in growth cones — produced decreased axonal extension rates.

Analysis of the frequency of calcium transients produced by growth cones at a guidance choice point — the point at which the direction of axonal growth changes — indicated that transients may be important not only for axonal extension but also for guidance. The axons of spinal motor neurons and interneurons initially extend perpendicularly towards the ventrolateral funiculus (VLF) of the spinal cord, but then the axons make sharp turns in order to extend along the VLF (Figure 1b). Gomez and Spitzer [8] reported that the growth cones of axons approaching the VLF at near-perpendicular angles transiently stopped at the VLF entry point before turning to extend along the VLF. While stopped at the VLF entry point, growth cones exhibited a high frequency of intracellular calcium transients (Figure 1b). Interestingly, other growth cones that approach the VLF at shallow angles did not stop at the VLF entry point and did not exhibit increased frequencies of calcium transients. These data demonstrate that calcium transients in growth cones *in vivo* are a major determinant of axonal extension rates and provide intriguing insight into a possible role for calcium transients at choice points during growth cone guidance. The dramatic change in direction of motor neuronal growth cones at the VLF may be promoted by an increased remodeling of the cytoskeleton induced by the calcium transients [9].

The studies reviewed in this article provide us with great insights into the variety of mechanisms that regulate axonal pathfinding *in vivo*. During axonal guidance, extracellular cues must be translated into alterations in the organization of the growth cone cytoskeleton. These studies contribute to our understanding of axonal guidance by describing how interactions between kinases and phosphatases and the dynamics of calcium signaling relate to growth cone navigation (Figure 2). A number of issues require further study, however. For example, what are the extracellular cues that activate Abl and Dlar? Are there different signals that activate, in a competing manner, either kinases or phophatases? If so, how are these signals integrated? What signals and transduction mechanisms regulate the frequency of calcium transients in growth cones? What are the effects of preventing calcium transients during growth cone guidance at choice points? Future studies, *in vitro* and *in vivo*, will surely provide us with an even greater appreciation of the signaling pathways and subsequent cytoskeletal rearrangements that are responsible for axonal guidance.

Acknowledgements
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